

IN THE SPECIFICATION:

Please replace paragraph number [0018] with the following rewritten paragraph:

B1 [0018] The average titer of antibody against IBDV in a flock will decline in time (~~Fig. 1~~FIG. 1). As a result of the decrease in average antibody titers, an immunity gap will occur. The best results are obtained if the immunity gap is as short as possible and is as early as possible, within a minimum of 2 weeks after hatching. There should be at least sufficient immunity after active vaccination at the age of 4 weeks, since many handlings occur in the houses from that time point with risks of introducing field virus. Therefore, farmers like to vaccinate at 2 weeks or even before. Intermediate vaccines are often unable to break through the average IBDV antibody titer of the broiler at two weeks after hatching (~~Fig. 1~~FIG. 1). If there is a high variation in mean maternal antibody titers, some chicks will be effectively vaccinated with intermediate vaccines, others not. To circumvent those problems, hot vaccines are being used. A drawback of usage of hot vaccines is that the bursa of chickens with low-to-moderate maternal antibody titers will be (partly) damaged.

Please replace paragraph numbers [0044] through [0074] with the following rewritten paragraphs:

B2 [0044] ~~Fig. 1~~FIG. 1: Antibody titres in broilers having high levels of maternal antibody at day 0.

[0045] ~~Fig. 2a: FIGs. 2A-2G:~~ Alignment of IBDV A-segment cDNA sequences CEF94-A (SEQ ID NO:71), D6948-A (SEQ ID NO:72), TY89-A (SEQ ID NO:73) and consensus sequence (SEQ ID NO:70).

[0046] ~~Fig. 2b: FIGs. 3A-3F:~~ Alignment of IBDV B-segment cDNA sequences CEF94-B (SEQ ID NO:75), D6948-B (SEQ ID NO:76) and consensus sequence (SEQ ID NO:74).

[0047] ~~Fig. 3a: FIGs. 4A-4C:~~ IBDV polyprotein alignment for CEF94-PP (SEQ ID NO:78), D6948-PP (SEQ ID NO:79), TY89-PP (SEQ ID NO:80) and consensus sequence (SEQ ID NO:81).

[0048] ~~Fig. 3b:~~ FIGs. 5A-5B: IBDV VP1 alignment for CEF94-VP1 (SEQ ID NO:82), D6948-VP1 (SEQ ID NO:83) and consensus sequence (SEQ ID NO:81).

[0049] ~~Fig. 3c:~~ FIG. 6: IBDV VP5 alignment for D6948-VP5 (SEQ ID NO:85), CEF94-VP5 (SEQ ID NO:86) and consensus sequence (SEQ ID NO:84).

[0050] ~~Fig. 4:~~ FIG. 7: Plasmid drawings.

[0051] ~~Fig. 5a:~~ FIG. 8A: Construction of pHB36-vvVP2.

[0052] ~~Fig. 5b:~~ FIG. 8B: Construction of pHB36-vvVP3.

[0053] ~~Fig. 5c:~~ FIG. 8C: Construction of pHB36-vvVP4.

[0054] ~~Fig. 5d:~~ FIG. 8D: Construction of pHB36-s2VP3.

[0055] ~~Fig. 5e:~~ FIG. 8E: Construction of pHB36-s2VP3C.

[0056] ~~Fig. 5f:~~ FIG. 8F: Construction of pHB36-s2VP3N.

[0057] ~~Fig. 5g:~~ FIG. 8G: Construction of pHB60-s2VP3C1.

[0058] ~~Fig. 6:~~ FIG. 9: Comparison of the amino acid sequences of the VP3-encoding parts of the cDNAs of the cell culture adapted serotype I classical strain CEF94 (amino acids 724-1012 of SEQ ID NO:78) and the wild-type serotype II strain TY89 (amino acids 2-290 of SEQ ID NO:80). The sequence of the CEF94 protein is presented, while only those amino acids, which differ from the CEF94 sequence, are given for TY89. Identical amino acids are represented by a dot. Plasmid pHB36-s2VP3 encodes the entire given amino acid sequence of serotype II (TY89). The nucleotide sequences of the A-segment cDNAs, which were used to deduce the amino acid sequences, have been deposited in the GenBank database: A-segment CEF94 (AF194428, full-length) and TY89 (xxxxx, partially). The positions of the *Sac*II and *Sca*I sites in the corresponding cDNA (see ~~Fig. 7~~ FIG. 10) are indicated.

[0059] ~~Fig. 7:~~ FIG. 10: Schematic representation of plasmids containing the full-length serotype I (CEF94) IBDV A-segment cDNA (pHB-36W), and mosaic full-length A-segment cDNAs (pHB36-s2VP3N, -s2VP3C and -s2VP3) based upon the genome of serotype I (CEF94) and serotype II (TY89) IBDV strains. Open boxes represent open reading frames based upon serotype I cDNA, while shaded boxes represent serotype II cDNA. Several genetic elements such as the remaining part of the polyprotein, the VP5 ORF, T7 RNA polymerase promoter and

terminator sequences and the Hepatitis Delta Virus Ribozyme (HDVR) are only shown for pHB-36W but these are also present at the same positions in the other plasmids.

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[0060] ~~Figs. 8A, 8B and 8C~~: FIGs. 11A, 11B, and 11C: SDS-PAGE analysis of IBDV proteins. A) Autoradiogram of a coupled *in vitro* transcription/translation reaction using plasmids containing either the full-length A-segment sequence of CEF94 (lane 1: pHB-36W) or mosaic full-length A-segment sequences (lane 2: pHB36-s2VP3, lane 3: pHB36-s2VP3N, or lane 4: pHB36-s2VP3C). The positions of the viral (precursor) proteins are indicated on the right-hand side, while the position of marker proteins (<sup>14</sup>C-labelled Amersham Rainbow marker) and their sizes (kDa) are indicated on the left hand side. B) Western blot analysis of purified IBDV of serotype I (lane 1: CEF94) and serotype II (lane 2: TY89). VP3 proteins (indicated by arrows) were visualized using a VP3-specific monoclonal antibody. The position of marker proteins (pre-stained Amersham Rainbow marker) and their sizes (kDa) are indicated on the right-hand side. C) Autoradiogram of immunoprecipitated <sup>35</sup>S-methionine-labeled IBDV proteins. *In vitro* synthesized viral proteins originating from pHB-36W (lane 1), pHB36-s2VP3 (lane 2), pHB36-s2VP3N (lane 3), or pHB36-s2VP3C (lane 4) were immunoprecipitated using either a polyclonal antibody directed against serotype I VP3 and VP4 (PaVP3/4), or monoclonal antibodies specific for VP3 of either serotype I (Mab IV<sup>SI</sup>) or serotype II (Mab VII<sup>SI</sup>).

[0061] ~~Fig. 9~~: FIG. 12: Single-step growth curves of rCEF94 and mCFE94-s2VP3C. QM5 cells were infected with IBDV (m.o.i. = 5, T = 0h) for one hour, washed four times, and covered with fresh medium. Samples were taken from the supernatant at different time points and the amount of IBDV (TCID<sub>50</sub> per ml) was determined. Error bars represent standard deviations.

[0062] ~~Fig. 10~~: FIG. 13: Detection of VP3 antigen in (m)IBDV-infected QM5 cells using different monoclonal antibodies. Fresh monolayers of QM5 cells were infected with either rCEF94, mCEF94-s2VP3, -s2VP3N, or -s2VP3C and incubated during 48h. Different monoclonal antibodies specific for VP3 of IBDV were used to assess the reactivity with the different mosaic viruses in an IPMA. An overview of the IPMA results of all used monoclonal antibodies is given in Table 11.

[0063] ~~Fig. 11:~~ FIG. 14: Schematic representation of the plasmids containing the full-length (hybrid) A-segment cDNA sequences. The T7 promoter sequence, the hepatitis delta virus ribozyme (HDR), and the T7 terminator are only given for plasmids pHB36W and pHB60, but is present in each plasmid. Open boxes indicate CEF94 cDNA; shaded boxes indicate D6948 cDNA, while black boxes indicate TY89 cDNA.

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[0064] ~~Fig. 12:~~ FIG. 15: Western blot analysis of protein samples isolated 24h after transfection of FPT7-infected QM-5 cells with either pHB-36W (1), pHB36-s2VP3 (2), pHB36-s2VP3N (3), pHB36-s2VP3C (4), pHB-60 (5), pHB60-s2VP3N (6), pHB60-s2VP3C1 (7), pHB60-s2VP3C3 (8). Four SDS-PAGE gels, containing equal amounts of cell extracts, were blotted onto nitrocellulose, and VP3 was detected using the four indicated Mabs. Size markers (Rainbow marker, Amersham) are indicated in kDa on the left.

[0065] ~~Fig. 13:~~ FIG. 16: Comparison of the amino acid sequence of VP3 of the classical, cell culture adapted serotype I CEF94 strain (amino acids 724-1012 of SEQ ID NO:78), the very virulent serotype I D6948 strain (amino acids 724-1012 of SEQ ID NO:79) and the wild-type serotype II TY89 strain (amino acids 2-290 of SEQ ID NO:80). The sequence of the CEF94 protein is presented, while only those amino acids that differ from the CEF94 sequence are given for D6948 and TY89. The positions of the *Sac*II and *Sca*I endonuclease restriction sites in the corresponding cDNA (see ~~Fig. 11~~ FIG. 14) are indicated.

[0066] ~~Fig. 14:~~ FIG. 17: Formalin-fixed bursa sections stained with Hematoxylin-Eosin (H & E). Layer-type SPF chickens infected with the indicated virus (upper right corner) at day 21 were euthanized at day 13 post-infection, and bursas were examined. The overall damage of the follicular structure was determined according to Bayyari et al. (1996) and is given in Table 13.

[0067] ~~Fig. 15:~~ FIG. 18: Resulting data of the competition Elisa's. The CEF94 VP3 antigen was coated, and a diluted serum sample (taken at 13 days post-infection) of chickens infected with the indicated virus was mixed with either Mab IV<sup>SI</sup> or I/G4<sup>SI</sup>. The amount of bound Mab was determined using Rabbit-anti-Mouse conjugated with peroxidase and a TMB as substrate.

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[0068] ~~Figs. 16A, 16B and 16C:~~ FIGs. 19, 20A, 20B, 21A, and 21B: Amino acid comparison between the different ORFs of the cDNAs of wild-type vvIBDV isolate D6948 and the cell culture adapted classical isolate CEF94. The complete sequence of the D6948 proteins is given, while only those amino acids which differ from the D6948 sequence are given for CEF94 (below the D6948 sequence). The nucleotide sequences of the A- and B-segments, which were used to deduce the amino acid sequences, can be found in the GenBank database (accession numbers are given between parenthesis: A-segment D6948 (AF240686), CEF94 (AF194428), B-segment D6948 (AF240687), and CEF94 (AF194429). A) Amino acid sequence encoded by the first ORF (VP5) of the A-segments for D6948-VP5 (SEQ ID NO:87) and CEF94-VP5 (SEQ ID NO:86). B) Amino acid sequence encoded by the second ORF (polyprotein) of the A-segment for D6948-PP (SEQ ID NO:79) and CEF95-PP (SEQ ID NO:78). The VP4 of the polyprotein (underlined) is preceded by pVP2, while VP3 is located at the C-terminus (see ~~Fig. 17~~ Fig. 22). The putative cleavage sites between pVP2 and VP4, and between VP4 and as suggested by (18) have been used. Only recently it was shown that the actual cleavage sites are most likely located between amino acids 512-513 (pVP2-VP4) and 755-756 (VP4/VP3) (27). C) Amino acid sequence encoded by the single ORF encoded by the B-segment (VP1/VPg) for D6948-VP1 (SEQ ID NO:83) and CEF94-VP1 (SEQ ID NO:82). Dashes are given in the case where corresponding amino acids are missing. Amino acid changes which are found in all vvIBDV sequences are given in bold face. Amino acid residues that are reported to be involved in adaptation to non-B-lymphoid cells are given in italics.

[0069] ~~Fig. 17:~~ FIG. 22: Schematic representation of the plasmids containing the full-length cDNA sequence of the A-segment (pHB-60) and B-segment (pHB-55) of the wild-type very virulent IBDV isolate D6948. The cDNA sequence is preceded by a T7 promoter sequence and is followed by the hepatitis delta virus ribozyme (HDR), and a T7 terminator. The different ORFs are represented by open boxes.

[0070] ~~Figs. 18a, 18b and 18c:~~ FIGs. 23A, 23B, and 23C: Autoradiogram of an SDS-PAGE analysis of a coupled *in vitro* transcription/translation reaction. a) Full-length A-segment plasmids of attenuated classical IBDV isolate CEF94 (lane 1) and wild-type very

virulent IBDV isolate D6948 (lane 2). b) Full-length B-segment plasmids of CEF94 (lane 1) and D6948 (lane 2). c) Full-length A-segment CEF94 (lane 1) and full-length A-segment plasmid of the classical attenuated IBDV isolated (pHB-36) in which either pVP2 (pHB36-vvVP2, lane 2), VP3 (pHB36-vvVP3, lane 3), or VP4 (pHB36-vvVP4, lane 4) has been exchanged. The positions of the viral proteins are indicated on the right. The sizes of the marker proteins (Rainbow marker, Amersham) are indicated on the left.

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[0071] ~~Fig. 19:~~ FIG. 24: Detection of IBDV using the VP3/4 polyclonal antiserum. Samples of supernatant of different plasmid transfections were used to infect QM5 or primary bursa cells. After infection, the QM5 cells were incubated for 24h, while the primary bursa cells were incubated for 48h. IBDV proteins were visualized by performing an Immunoperoxidase Monolayer Assay.

[0072] ~~Fig. 20:~~ FIG. 25: Single-step growth curves of CEF94, rCEF94 and srIBDV-CADB. QM5 cells were infected with IBDV (m.o.i. = 5, T = 0h) for one hour, washed three times, and covered with fresh medium. At different time points, samples were taken from the supernatant and the amount of IBDV (TCID<sub>50</sub> per ml) was determined. The TCID<sub>50</sub> value at each time point is the mean of three independent experiments; error bars represent standard deviations.

[0073] ~~Fig. 21:~~ FIG. 26: Schematic representation of the construction of a mosaic PCR fragment having a middle part originating from D6948 cDNA (shaded boxes), and flanking parts originating from CEF94 cDNA (open boxes). For the construction of pHB-36-vvVP2, we first generated PCR fragments VP2a, VP2b, and VP2c. These PCR fragments were purified and subsequently used as template in a fusion PCR, yielding PCR fragment VP2d (17). This PCR fragment was subsequently purified and used to replace the corresponding part of the CEF94 A-segment cDNA, by using the indicated *EcoRI* and *SacII* restriction sites. pHB36-vvVP3 and -vvVP4 were generated using the same approach using different primers to generate the PCR fragments and different restriction sites to introduce the PCR fragments in the full-length A-segment clone (see Material and Method section and Table 16).

[0074] ~~Fig. 22:~~ FIG. 27: Detection of mosaic IBDV using VP3/4 polyclonal antiserum.

B2 Samples of supernatant of different plasmid transfections were used to infect QM5 and primary bursa cells. After infection, the QM5 cells were incubated for 24h, while the primary bursa cells were incubated for 48h (see ~~Fig. 19~~ FIG. 24). In contrast to the negative control (mock-infected), in which no B-lymphoid cells stained positive (data not shown), we found several stained B-lymphoid cells scattered throughout the culture tissue dish in the IPMA shown in the lower panel.

Please replace paragraph number [0080] with the following rewritten paragraph:

B2 [0080] The full-length PCR fragments which were generated three times independently from genomic dsRNA were isolated from the agarose gel by using a Qiaex gel purification kit (Qiagen) and ligated in the pGEM-Teasy (Promega) vector according to the suppliers instructions. The ligated plasmids were used to transform *E. coli* DH5-alpha cells which were subsequently grown under ampicillin selection (100 µg/ml) and in the presence of IPTG (0.8 mg per petri-dish)) and Bluo-gal (0.8 mg per petri-dish). Plasmid DNA of white colonies was prepared and analyzed by restriction enzyme digestion and agarose gel separation. The nucleotide sequences of the cloned cDNAs were determined by using an ABI310 automated sequencer and A- and B-segment-specific primers. The consensus nucleotide sequences of both segments of CEF94 and of both segments of D6948 were determined (~~Fig. 2~~) (FIGs. 2A-2G and 3A-3F) and the corresponding amino acid sequence of the open reading frames was deduced (~~Fig. 3~~) (FIGs. 4A-4C, 5A, 5B and 6). By using the cDNA of two independent clones, we restored one amino acid mutation present in the A-segment clone (V542A), resulting in pHB-36W, one amino acid mutation in the A-segment clone of D6948 (P677L), resulting in pHB-60, and one amino acid mutation in the B-segment of D6948 (Q291X), resulting in pHB-55. No amino acid mutations were present in the B-segment cDNA clone of CEF94 (pHB-34Z).

Please replace paragraph numbers [0084] through [0086] with the following rewritten paragraphs:

B4 [0084] For the construction of pHB36-vvVP2 (exchange of pVP2-encoding part, Table 4) we have used IBDV-specific primers to generate the mosaic PCR-VP2D fragment (2256 bp, see ~~Fig. 5a~~ FIG. 8A). The internal part of this PCR fragment was used to exchange the corresponding part of pHB-36W, using unique sites for restriction enzymes *EcoRI* and *SacII*.

[0085] For the construction of plasmid pHB36-vvVP3 (Table 4) we used IBDV-specific primers to generate a mosaic PCR-VP3c fragment (2154 bp, see ~~Fig. 5b~~ FIG. 8B). The internal part of this PCR fragment was used to exchange the corresponding part of pHB-36W, using unique sites for the *EagI* and *KpnI* (genetic tag site) restriction enzymes.

[0086] For the construction of plasmid pHB36-vvVP4 (Table 4) we used IBDV-specific primers to generate a mosaic PCR-VP4d fragment (2154 bp, see ~~Fig. 5c~~ FIG. 8C). The internal part of this PCR fragment was used to exchange the corresponding part of pHB-36W, using the unique site for restriction enzymes for *EagI* and *DraIII*.

Please replace paragraph number [0088] with the following rewritten paragraph:

B5 [0088] To obtain the cDNA of the A-segment of a serotype II IBDV isolate we generated single-stranded cDNA of TY89 as described above, by using the ANC1 primer. The coding region of the VP3 protein was subsequently independently amplified three times in a PCR by using 2 ml of RT-material, 1\* Taq buffer, 50  $\mu$ M of each dNTP, two IBDV serotype II-specific primers (0.2 pMol each), 1.5 units of enzyme, and 3.0 mM  $MgCl_2$  in a 0.1 ml reaction volume. Amplification was performed by cycling 35 times between 94° C (15 sec), 52° C (15 sec) and 72° C (1 min). The resulting 956 bp fragment was cloned in the pGEM-TEasy vector and the consensus nucleotide sequence was determined (~~Fig. 2a~~) (FIGs. 2A-2G). One of the isolated plasmids contained the TY89 VP3 consensus sequence (pSV-VP3-TY89, ~~Fig. 4~~) FIG. 7) and was used as template to generate an 893 bp PCR fragment (see ~~Fig. 5d~~ FIG. 8D). This PCR fragment was subsequently used to replace the corresponding part of plasmid pHB36W-vvVP3, by using the artificially introduced *KpnI* (nt 3175) and *SacII* (nt 1760)



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cont'd restriction sites in both plasmid pSV-VP3-TY89 and pHB36W-vvVP3. The resulting plasmid (pHB36-vvVP3, see Fig. 5d) FIG. 8D encodes the N-terminal 722 amino acids of the CEF94 polyprotein and the 290 C-terminal amino acids of the TY89 polyprotein. The intended exchange was confirmed by nucleotide sequence analysis.

Please replace paragraph number [0097] with the following rewritten paragraph:

Ble [0097] Using the sequence data of the 5'-termini, we cloned the entire coding and non-coding cDNA sequences of the A-segment and B-segment of classical isolate CEF94 by means of RT-PCR. Using the same procedure and using the same primers, we also generated the entire coding and non-coding cDNA of the A- and B-segments of a non-CEF-adapted, very virulent IBDV isolate D6948. The nucleotide sequence of the entire genome of both isolates was determined three times independently. This sequence information enabled us to generate a consensus nucleotide sequence of both the A- and B-segments of IBDV isolates CEF94 and D6948 ~~(Fig. 2a)~~ (FIGs. 2A-2G).

Please replace paragraph number [0099] with the following rewritten paragraph:

B7 [0099] To be able to generate IBDV from cloned cDNA which has the authentic terminal sequences, we introduced the cis-acting Hepatitis Delta Virus (HDV) ribozyme (Chowrira et al., 1994) downstream of the cDNA sequence of the A- and B-segments ~~(Fig. 4)~~ (FIG. 7). Furthermore, we introduced an additional modification in the 3' untranslated region of the CEF94 A-segment. By exchanging 2 nucleotides, we introduced a unique *Kpn*I endonuclease restriction site in this cDNA. The introduction of this unique restriction site enables us to distinguish between wild-type IBDV and infectious IBDV virus generated from cloned cDNA (genetically tagged rIBDV). As expected, this plasmid yields the same viral proteins in an *in vitro* transcription-translation reaction as the A-segment clone without the genetic tag (data not shown).

Please replace paragraph number [0103] with the following rewritten paragraph:

B8 [0103] Plasmid pHB-36 (A-segment CEF94, Table 4) contained a single nucleotide substitution at position 1875 (thymine instead of a cytosine) compared to the consensus CEF94 A-segment sequence (~~Fig. 2a~~). (FIGs. 2A-2G). This nucleotide substitution leads to a valine at position 582 of the polyprotein instead of an alanine, which is encoded by the consensus sequence (V582A, ~~Fig. 3a~~). (FIGs. 4A-4C). As this amino acid mutation is present in the viral protease (VP4), we subsequently checked whether this protease was still able to autocatalytically liberate the viral proteins (pVP2, VP3 and VP4) from the polyprotein. When plasmid pHB-36 was used as template in a coupled *in vitro* transcription/translation reaction in the presence of <sup>35</sup>S-labeled methionine, we found a delayed splicing of the polyprotein (data not shown). Apart from the viral proteins which are found in the case of normally spliced polyprotein (pVP2, VP3 and VP4), we found intermediate spliced products (60 kDa: VP4+VP3) and non-spliced polyprotein (data not shown). Although the viral protease (VP4) of clone pHB-36 is able to liberate the structural viral proteins (pVP2 and VP3) from the polyprotein, this clone did not yield rIBDV when using the FPT7-based transfection protocol as described above. Rapid autocatalytic cleavage of the polyprotein is apparently necessary for the generation of infectious rIBDV. We expect that other mutations within VP4 which alter the rate or specificity of the autocatalytic cleavage of the polyprotein will also have a negative effect on viability of the generated rIBDV. Furthermore, mutations in the region of the cleavage sites (pVP2-VP4 and VP4-VP3) may also have a negative effect on replication of rIBDV. Any mutation, introduced by modern molecular biological techniques into the cDNA of a very virulent IBDV may enable us to generate rIBDV which has a reduced viability and which can be used as a live or killed IBDV vaccine.

Please replace paragraph number [0108] with the following rewritten paragraph:

B9 [0108] The coding sequence of the C-terminal part of serotype II VP3 (122 amino acids) was also used to replace the corresponding part of the cDNA of D6948. During the exchange, we have replaced some D6948 cDNA sequence (encoding for the C-terminal part of

B9 cont'd  
 VP4 and the N-terminal part of VP3, and the 3'-UTR) with the corresponding sequence of CEF-94 (see Fig. 5g). FIG. 8G). The resulting plasmid (pHB60-s2VP3C1) was, together with pHB-55 (B-segment D6948), transfected into FPT7-infected QM5 cells. Supernatant of these transfected QM5 cells was collected after 24 h and was transferred to embryonated eggs and primary bursa cells. By using monoclonal antibodies, we were able to detect infected cells in the monolayer of primary bursa-derived cells (see Table 5). mD6948-s2VP3C1 gave the same reaction pattern with the monoclonal antibodies as mCEF-s2VP3C did. Isolate mD6948-s2VP3C1 (1000 ELD50/chicken) was also used to infect 10 SPF chickens (21 days old) to evaluate its virulence. This mIBDV isolate did not cause any mortality in a 9-day course, opposite to the D6948, rD6948 and srIBDV-DACB isolates (Table 6). However, the bursa is severely damaged by this mIBDV, as the bursa-body weight ratio of this group is the same as found in the groups which received D6948 or rD6948. This indicates that mD6948-s2VP3C1 is still able to replicate and induce apoptosis in the bursa of Fabricius.

Please replace paragraph number [0116] with the following rewritten paragraph:

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 [0116] The generation of pUC19-based plasmids containing the full-length cDNA of the two genomic dsRNA segments of CEF94 has been described before. The cDNA in these plasmids is preceded by a T7-promoter sequence and followed by the antigenomic Hepatitis Delta Virus ribozyme (Been, Perrotta, and Rosenstein, 1992) and a T7 terminator sequence. The entire VP3-encoding part of the serotype II strain TY89 was cloned by performing an RT-PCR on the dsRNA of TY89. The reverse transcription was performed with primer ANC1 (GGGGACCCGCGAACGG (SEQ ID NO:2)). Three independent PCRs were performed using primers RTAM (AATTGGTGTCCACACCTG (SEQ ID NO:3)) and RTAP (ATACAGGACCTAACTGGG (SEQ ID NO:4)) and 35 amplification cycles (annealing temperature of 52° C, and *Taq* polymerase). The resulting fragments (956 bp) were separately cloned into the pGEM-T Easy vector (Promega). The nucleotide sequence was determined on both strands using specific primers in a cycle sequencing reaction (BigDye terminator kit, PE Applied Biosystems) and an ABI310 DNA sequencer (PE Applied Biosystems). The consensus

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would nucleotide sequence of the VP3 cDNA of TY89 was determined and the amino acid sequence (Fig. 6) ~~(FIG. 9)~~ was deduced. One of the clones, pSV-VP3-TY89(1), contained the consensus sequence and was used to generate the mosaic A-segment cDNA clones (see below).

Please replace paragraph number [0117] with the following rewritten paragraph:

B11 [0117] To replace VP3 of CEF94 with VP3 of TY89 in the full-length A-segment clone of CEF94, we amplified the TY89 VP3-encoding part by PCR using primers HY3P (AACGTTTTCTCACA TCCgCGgGACTGGG (SEQ ID NO:5), non-hybridizing nucleotides are given as lowercase letters) and AGTM (GAGACTCCCAGGtaCCTCACTC (SEQ ID NO:6)) (annealing temperature is 58° C, *Pwo* polymerase, and 30 cycles). This PCR fragment (893 bp) was digested with *Sac*II (2316) and *Kpn*I (nt 3172), resulting in a 856 bp fragment, which was agarose gel purified (Qiaex gel extraction kit, Qiagen). The fragment was subsequently used to replace the corresponding part of plasmid pHB36-vvVP3, which had been digested with *Sac*II (partially, nt 2316) and *Kpn*I (nt 3172). After ligation of the PCR fragment into the vector (Rapid ligation Kit, Roche Molecular Biochemicals), we transformed *E. coli* DH5a cells with this mixture. Plasmids obtained from several independent colonies were analyzed and one plasmid having the intended sequence as determined by sequencing analysis was selected (pHB36-s2VP3, see Fig. 7) ~~FIG. 10~~.

Please replace paragraph number [0121] with the following rewritten paragraph:

B12 [0121] For *in vitro* transcription and translation, we used circular plasmids (0.4 µg) containing full-length A- or B-segments (see ~~Fig. 7~~ FIG. 10) in a 2.5 ml TnT-T7Quick reaction (Promega) in the presence of <sup>35</sup>S-methionine. The resulting viral proteins were separated either directly or after immunoprecipitation in an SDS-PAGE gel (12%) and detected by autoradiography. For immunoprecipitation of *in vitro*-produced proteins, we used either polyclonal (PaVP3/4) or monoclonal antibodies directed against VP3 of serotype I IBDV (Mab IV<sup>SI</sup>) or serotype II (Mab VII<sup>SI</sup>). RIPA buffer (Sambrook, Fritsch, and Maniatis, 1989) was used during all incubations and washing steps. Antigen-antibody complexes were purified from the

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mixture by addition of Protein A Sepharose CL-4B (Amersham Pharmacia, Sweden), and three washing steps were performed.

Please replace paragraph numbers [0125] through [0127] with the following rewritten paragraphs:

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[0125] To construct serotype I recombinant IBDV viruses containing the VP3 protein of serotype II, we had to clone the corresponding cDNA of a serotype II strain. First, we attempted to clone the full-length A- and B-segment cDNAs of TY89, the prototype serotype II IBDV strain (McFerran et al., 1980). To generate these cDNAs, we followed the full-length RT-PCR protocol that we used to clone the A-segment of serotype I strains. Although we succeeded repeatedly in cloning the full-length B-segment cDNA of TY89 (data not shown), we were unable to obtain the full-length cDNA of the A-segment using this protocol. Therefore, we only amplified the VP3-encoding part of the TY89 A-segment. An RT-PCR fragment containing VP3 of TY89 was obtained three times independently and cloned in the pGEM-T Easy vector. The consensus sequence for this part of the TY89 genome was determined and the deduced amino acid sequence was compared with the corresponding sequence of CEF94, a cell culture adapted serotype I strain (~~Fig. 6).~~ (FIG. 9). The cDNA encoding the TY89 VP3 was subsequently used to generate mosaic A-segment cDNA plasmids which encode either the complete VP3 of TY89 (pHB36-s2VP3) or the N-terminal part (101 amino acids, pHB36-s2VP3N) or the C-terminal part (155 amino acids, pHB36-s2VP3C) (~~Fig. 7).~~ (FIG. 10).

[0126] After having verified that the obtained plasmids had the correct sequence, they were used as templates in an *in vitro* transcription/translation reaction, followed by SDS-PAGE and autoradiography (~~Fig. 8A).~~ (FIG. 11A). The three proteins, pVP2, VP3 and VP4, which result from autocatalytic cleavage of the polyprotein, were present in all cases. The positions of the VP3 proteins derived from plasmids pHB36-s2VP3 and pHB36-s2VP3N were found at a higher position in the SDS-PAGE gel than VP3 of pHB-36W and pHB36-s2VP3C. A difference in migration during SDS-PAGE separation was also found between viral VP3 obtained from purified CEF94 and TY89 virus in a western blot analysis (~~Fig. 8B).~~ (FIG. 11B). This indicates

that the observed difference in migration of VP3 from the mosaic full-length A-segment plasmids is not an experimental artifact, but due to amino acid differences in the N-terminal part of VP3.

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[0127] Three amino acid changes are present in the viral protease (VP4) of pHB-36W-s2VP3 and -s2VP3N (~~Fig. 6~~), (~~FIG. 9~~), which is due to the fact that the suggested cleavage site of the polyprotein (Hudson et al., 1986) appeared not to be the genuine cleavage site as recently has been shown (Sanchez and Rodriguez, 1999). These differences apparently do not alter the migration behavior of VP4 in an SDS-PAGE analysis, as this VP4 is found at the same position as wild-type serotype I VP4 (~~Fig. 8A~~), (~~FIG. 11A~~). Also, no influence of these amino acid changes was found on the *in vitro* cleavage of the polyprotein, as no uncleaved or partially cleaved polyprotein was present (~~Fig. 8A~~), (~~FIG. 11A~~).

Please replace paragraph number [0128] with the following rewritten paragraph:

B14

[0128] Immunodominant epitopes, recognized by non-neutralizing antibodies, are present on VP3. At least one of these epitopes is serotype specific as has been reported by several researchers (Mahardika and Becht, 1995; Oppling, Muller, and Brecht, 1991; Reddy, Silim, and Ratcliffe, 1992). To map the position of serotype-specific epitopes and to assess their usefulness for discriminating virus rescued from mosaic A-segment cDNAs, we performed a radio-immune-precipitation (RIP) of the *in vitro*-translated and  $\{^{35}\text{S}\}$ -Met-labeled VP3 proteins, and used either a polyclonal (PaVP3/4) or monoclonal (Mab IV<sup>SI</sup> or Mab VII<sup>SI</sup>) antibody in the RIP. We analyzed the precipitated proteins by SDS-PAGE, followed by autoradiography (~~Fig. 8C~~), (~~FIG. 11C~~). As expected from the high level of homology, VP3 derived from both serotypes I and II was precipitated by polyclonal PaVP3/4 serum. However, monoclonal antibody IV<sup>SI</sup> only precipitated VP3 that contained the C-terminal part of serotype I VP3, while Mab VII<sup>SI</sup> precipitated solely VP3 that contained the C-terminal part of serotype II VP3. These results indicate that a serotype-specific epitope is present in the C-terminal 155 amino acids of VP3.

Please replace paragraph number [0130] with the following rewritten paragraph:

B15 [0130] To determine the effect of the exchange of parts of the polyprotein on replication, we determined the maximum titers of each virus after three serial passages. Although the mosaic virus which contained 3 amino acid changes in VP4, in combination with the N-terminal part of the serotype II VP3, did show an increase in viral titers after three serial passages, the maximum TCID<sub>50</sub> values (6.0) obtained were about 10-fold lower than those of unmodified rCEF94 and of mosaic virus containing the C-terminal part of serotype II VP3 (Table 10). To investigate whether rCEF94 and mCEF94-s2VP3C, which were rescued with the same efficiency, had the same replication kinetics in cell culture, we performed a single-step growth curve with both viruses using QM5 cells. Although the maximum titers obtained were the same at 25h post-infection (p.i.), there was a delay in virus release in the case of mCEF94-s2VP3C in comparison to unmodified rCEF94 (~~Fig. 9).~~ (FIG. 12). The first virus release after infection with unmodified rCEF94 was found at 10h p.i., while at that time point, no release of virus was found after infection with mCEF94-s2VP3C.

Please replace paragraph number [0131] with the following rewritten paragraph:

B16 [0131] After rescue of the mosaic IBDV, we assessed the possibility of discriminating between these IBDVs by using a set of VP3-specific monoclonal antibodies. Fresh QM5 cells were infected with the rescued viruses rCEF94, mCEF94-s2VP3, -s2VP3N, and -s2VP3C, and viral antigens were detected at 48h p.i., by using IBDV-specific monoclonal antibodies. Viral antigen originating from all the four different rescued viruses was detected in infected QM5 cells when serotype I-specific neutralizing VP2 antibodies (1.4 or 9.8, see Table 11) or non-neutralizing non-serotype-specific VP3 antibodies (9.7 or 17/80, see Table 11) were used. On the other hand, VP3 monoclonal antibodies which are serotype specific reacted differently with the four rescued viruses. Serotype I-specific antibodies (IV<sup>SI</sup> or I/G4<sup>SI</sup>, ~~see Fig. 10~~ FIG. 13) reacted only with rCEF94 and mCEF94-s2VP3N while the serotype II-specific antibody (VII<sup>SI</sup>, ~~see Fig. 10~~ FIG. 13) reacted only with mCEF94-s2VP3 and mCEF94-s2VP3C. The reaction pattern of the monoclonal antibodies used was the same when tested in an RIP on *in vitro*

synthesized IBDV proteins (~~Fig. 8C~~) (FIG. 11C) and by an IPMA on infected QM5 monolayers (~~Fig. 6~~) (FIG. 9).

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Please replace paragraph number [0134] with the following rewritten paragraph:

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97  
[0134] First, the VP3-encoding cDNA of TY89 was cloned by means of RT-PCR and its nucleotide sequence was determined (see ~~Fig. 6~~ FIG. 9 for the deduced TY89 amino acid sequence). This cDNA was subsequently used to generate mosaic full-length A-segment cDNA plasmids (~~see Fig. 7~~) (see FIG. 10). These full-length plasmids were analyzed for the production of viral proteins in a combined *in vitro* transcription/translation reaction in the presence of  $\{^{35}\text{S}\}$ -labeled methionine. Autocatalytic cleavage of the polyprotein by VP4 was not affected by the (partial) exchange of VP3. Also, the exchange of three amino acids of serotype I VP4, just in front of the VP4-VP3 cleavage site of the polyprotein in plasmids pHB36-s2VP3 and -s2VP3N (see ~~Fig. 6~~) (FIG. 9), did not influence the autocatalytic cleavage. On the other hand, a quite remarkable difference in migration in SDS-PAGE between the *in vitro* synthesized VP3 originating from either serotype I or serotype II cDNA (~~Fig. 8A~~) (FIG. 11A). The same difference in electrophoretic mobility was found for VP3 of purified TY89 and CEF94 (~~Fig. 8B~~) (FIG. 11B), although the predicted sizes of these proteins are similar (28.8 kDA). A similar difference in migration behavior between VP3 derived from serotype I and II IBDV has also been noted by others (Oppling, Muller, and Brecht, 1991; Reddy, Silim, and Ratcliffe, 1992). Furthermore, we observed two VP3 bands (VP3a and VP3b) in the Western blot analysis of purified CEF94 and TY89 (~~Fig. 8B~~) (FIG. 11B). Whether these two VP3 bands both represent functional proteins, or whether the smaller band (VP3b) is just a specific degradation product of the larger band (VP3a), is unclear. We have observed the presence of two VP3 bands for cell culture amplified CEF94 previously. Fahey et al. (Fahey, Erny, and Crooks, 1989) have made a similar observation for the Australian IBDV isolate 002/73, while also the presence of two VP3 bands for IPNV has been reported (Dobos, 1977) (Hjalmarsson and Everitt, 1999).

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Please replace paragraph number [0136] with the following rewritten paragraph:

B18 [0136] The first reports of a potential IBDV marker vaccine virus concerned a VP5 knock-out mutant of the attenuated, cell culture adapted rD78 vaccine strain (Mundt, Kollner, and Kretzschmar, 1997; Yao, Goodwin, and Vakharia, 1998). Although a VP5-minus rIBDV might yield a good serological marker, its efficacy as a vaccine has not been proven yet. Replication of VP5-minus rIBDV is severely affected both *in vitro* and *in vivo*. Mundt et al. (Mundt, Kollner, and Kretzschmar, 1997) reported that VP5-minus rD78 had a highly reduced virus release (> 200-fold lower titer after 24h p.i. in a single-step growth curve), while Yao et al. (Yao, Goodwin, and Vakharia, 1998) reported a large reduction in virus yield and a much higher survival rate of CEF cells (8 days p.i., multi-step growth curve), in comparison to unmodified rD78. Although mCEF94-s2VP3C has a slightly retarded release of virus (~~Fig. 9~~), FIG. 12, replication in cell culture is much less affected than that of the VP5 knock-out mutant. The finding that mCEF94-s2VP3C virus replicates almost as efficiently as an unmodified serotype I strain, in combination with the possibility to discriminate this virus from both serotype I and II wild-type field isolates, makes the use of the C-terminal part of serotype II VP3 a very promising approach to develop an IBDV marker vaccine.

Please replace paragraph numbers [0144] and [0145] with the following rewritten paragraphs:

B19 [0144] The construction of hybrid CEF94 and TY89 plasmids have been described before. For the construction of plasmid pHB60-s2VP3N, we generated 5 different PCR fragments. In all PCRs we have used *Pwo* polymerase during 20 reaction cycles. PCR-1N was made using pHB-60 as template and primers AC3 (GGTAGCCACA TGTGACAG (SEQ ID NO:7)) and HY3MR (CCAGTCCCGC GGATTGTGAGG (SEQ ID NO:8)) at 56° C, yielding a 1614 bp fragment (nt 731-2346). PCR-2N was made using pHB36-s2VPN as template and primers HY3P (AACGTTTTCC TCACAATCCG CGGGACTGGG (SEQ ID NO:5)) and M13F-17 (GTAAAACGAC GGCCAGT (SEQ ID NO:9)) at 56°C, yielding a 1251 bp fragment (nt

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2318-3566). PCR-3N was made using gel-purified PCR-1N and PCR-2N fragments as template and primers AC4 (ACCCAGCCAA TCACATCC (SEQ ID NO:10)) and AGTM (GAGACTCCCA GGTACCTCAC TC (SEQ ID NO:6)) at 54° C, yielding a 2151 bp fragment (nt 1057-3208), which was subsequently digested with *ScaI* (nt 2799), yielding PCR-3sN. PCR-4N was made using pHB-60 as template and primers AC9 (CTCAAAGAAG ATGGAGACC (SEQ ID NO:11)) and M13F-24 (CGCCAGGGTT TTCCCAGTCA CGAC (SEQ ID NO:12)) at 54° C, yielding a 869 bp fragment (nt 2727-3593). PCR-5 was made using gel-purified PCR-3sN and PCR-4 fragments as template and primers AC5 (AAGGCCTTCA TGGAGGTGGC CG (SEQ ID NO:13)) and M13F-17 (GTAAAACGAC GGCCAGT (SEQ ID NO:9)) at 54° C, yielding a 2143 bp fragment (nt 1423-3566), which was subsequently digested with *XhoI* and *XbaI*, yielding a 1889 bp PCR-5xxN fragment (nt 1606-3495). The PCR-5xxN fragment was subsequently used to replace the corresponding part of pHB-60 using the rapid DNA ligation kit and transformation of *E. coli* cells. The DNA sequence of the selected plasmid clone of pHB60-s2VP3N was determined (nt 1600-3325), and appeared to have the intended mosaic D6948-TY89 cDNA sequence as shown in Fig.1-FIG. 1.

[0145] For the construction of pHB60-s2VP3C3, we first transferred a 1735 bp fragment of pHB36-s2VP3C into pHB-60 using the unique *SacII* (1670) and *XbaI* (3495) sites, yielding pHB60-s2VP3C1. The 3'-UTR region of CEF94 was subsequently replaced by the corresponding cDNA sequence of D6948. A PCR fragment (385 bp, nt 3184 -3566) was generated by using primers AGTP (CTTGAGTGAG GTACCTGGGAG (SEQ ID NO:14)) and M13F-17 (GTAAAACGAC GGCCAGT (SEQ ID NO:9)), using pHB-60 as template, with a hybridization temperature of 52° C, *Pwo* polymerase, and 20 reaction cycles. This PCR fragment was purified, and digested with *KpnI* and *XbaI*. The resulting fragment (315 bp, nt 3198-3519) was gel purified and used to replace the corresponding part of pHB60-s2VP3C1 (digested with *KpnI* and *XbaI*), by using rapid ligation and transformation of *E. coli* cells, yielding pHB60-s2VP3C2. Next, a PCR fragment (1187 bp, nt 1423-2607) was generated by using primers AC5 (AAGGCCTTCA TGGAGGTGGC CG (SEQ ID NO:13)) and vvVP3CM (GAGAAAATTT CGCATCCGATG (SEQ ID NO:15)) using pHB-60 as template, with a

B19 cont'd  
hybridization temperature of 54° C, *Pwo* polymerase, and 20 reaction cycles. This PCR fragment was purified (High Pure PCR purification, Boehringer Mannheim), digested with *Sac*II (nt 1760) and *Apo*I (nt 2576). The resulting 816 bp fragment was used to replace the corresponding part of pHB60-s2VP3C2 (digested with *Apo*I (nt 2576, partially) and *Sac*II (nt 1760)), by using rapid ligation and transformation of *E. coli* cells, yielding pHB60-s2VP3C3. The DNA sequence of the selected plasmid clone of pHB60-s2VP3C3 was determined (nt 1600- 3275), and appeared to have the intended mosaic D6948-TY89 cDNA sequence as shown in Fig-1FIG. 1.

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Please replace paragraph number [0151] with the following rewritten paragraph:

B20  
[0151] To assess the viability, virulence and antigenic properties of IBD viruses containing a chimeric A-segment dsRNA, we have made several plasmids which contain a full-length A-segment cDNA encoding a hybrid polyprotein. Next to A-segment plasmids based upon CEF94 cDNA, and encoding a VP3 which is (partially) derived from the TY89 serotype II isolate, we constructed three chimeric plasmids which are based upon A-segment plasmid of D6948, a very virulent IBDV isolate. Two of these plasmids (pHB60-s2VP3C1 -s2VP3C3, see Fig-11- FIG. 14 and Table 12) encoded a polyprotein in which the C-terminal part of the VP3 is derived from serotype II, while the other (pHB60-s2VP3N, Fig-11- FIG. 14 and Table 12) encodes a polyprotein in which the N-terminal part of VP3 originates from serotype II IBDV. All plasmids encoding the hybrid VP3 proteins possess a T7 promoter and the hepatitis delta virus ribozyme. T7 RNA polymerase-driven transcription of these plasmids leads to positive stranded A-segment IBDV RNA which exactly mimics the nucleotide sequence of the viral positive stranded RNA. A difference is, however, present between native viral RNA and artificially produced viral-like RNA, because a viral protein genome-linked molecule (VP1) is present at the 5'-end of native RNA which lacks in the artificially produced RNA.

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Please replace paragraph number [0152] with the following rewritten paragraph:

B21  
[0152] The constructed plasmids were subsequently used to transfect QM5 cells, which were infected prior to transfection with a recombinant poxvirus (either Fowlpox (FP-T7 or

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vaccinia MVA-T7) which expresses T7 polymerase cytoplasmatically after infection. No replication of the resulting A-segment RNA can occur as the RNA-dependent RNA polymerase (VP1), encoded by the B-segment, is absent. After transfection, the cells were washed and the total cell extract was assessed for the presence of the (recombinant) VP3 by Western blot analysis ~~(Fig. 12).~~ (FIG. 15). Four different monoclonal antibodies known to react (serotype-specific) with VP3 were used. Monoclonal B10A is a non-serotype-specific monoclonal antibody and reacted with VP3 encoded by all the used plasmids. The serotype I-specific monoclonal IV<sup>SI</sup> reacts only with those VP3's which possess the C-terminal part of serotype I, while monoclonal I/G4<sup>SI</sup> only recognizes the VP3 C-terminal part of the cell culture adapted classical serotype I isolate (CEF94), and not with the very virulent serotype I- (D6948-) derived VP3. The recognition pattern of the serotype II-specific monoclonal T75<sup>SI</sup> is just the opposite of monoclonal IV<sup>SI</sup>; it recognizes exclusively those VP3's which possess the serotype II C-terminal part.

Please replace paragraph number [0153] with the following rewritten paragraph:

B22

[0153] Based upon the Western blot analysis, we concluded that the VP3 epitopes, which are recognized by the three serotype-specific Mabs, are linear and present in the C-terminal part (amino acid 850-1012) of this protein. An alignment of the deduced amino acid sequences of the VP3 part of the polyprotein reveals that only two amino acids are different between the two serotype I isolates CEF94 and D6948 (i.e., amino acid 981 and 1005; see ~~Fig. 13).~~ FIG. 16). The fact that monoclonal I/G4<sup>SI</sup> only recognizes CEF94 and not D6948 is apparently due to one of these amino acid differences. Furthermore, I/G4<sup>SI</sup> is also unable to react with TY89. Several differences are present between the VP3 of the serotype II and serotype I strains. Because the epitope for I/G4<sup>SI</sup> is present either around amino acid 981 or 1005 (see above), we concluded that the amino acid difference at position 981 or 992 (see ~~Fig. 13).~~ FIG. 16) is responsible for the difference in reactivity of I/G4<sup>SI</sup>.

Please replace paragraph number [0155] with the following rewritten paragraph:

623 [0155] To assess the virulence of rescued mosaic IBDV strains, we inoculated (internasally and intraocularly) groups of SPF chickens with 1000 ELD<sub>50</sub> of wild-type or mosaic virus strains and determined several relevant parameters, both during the living phase and post-mortem (Table 13). In the first animal experiment (Exp. 1), we observed mortality only in groups which received D6948 (either wild-type or rescued) during the first five days after inoculation. No mortality occurred in this experiment when the D6948 derivative mD6948-s2VP3C1 was used to inoculate the chickens. This is in contrast to the second animal experiment (Exp. 2) in which one chicken (out of 15) of this group died within 5 days p.i. In Exp. 2 we found no mortality after infection with mD6948-S2VP3C3, a derivative of D6849 which is closer to the wild-type D6948 than mD6948-s2VP3C1 (see ~~Fig. 11~~ FIG. 14 and Table 13). The bursa and body weights of each chicken were determined after euthanization (Table 13). The group average of the ratio between the bursa weight and body weight is a good indication of the damage of the bursa of the SPF chickens, due to the IBDV infection. Cell culture adapted IBDV strains (e.g., CEF94), which no longer possess a specific tropism for developing B-lymphoid cells in the bursa of Fabricius, induced only a minor reduction of this ratio (Table 13), while non-cell culture adapted IBDV strains (e.g., the very virulent D6948 and derivatives thereof), which possess the specific bursa tropism, induced a large reduction of the bursa/body weight ratio. The introduction of the C-terminal part encoding part of the serotype II VP3 into the genome of vvIBDV (mD6948-s2VP3C3) did not alter this ratio, consistent with the fact that tropism for the bursa correlates with the sequence of VP2. Despite the fact that the very virulent VP2 sequence is present in mD6948-s2VP3C1, a difference is found for the bursa/body weight ratio of mD6948-s2VP3C1 in comparison to wild-type or rescued D6948. This specific combination of the D6948, CEF94 and TY89 genomes apparently influences viral replication and hence bursa damage in this animal experiment (Exp. 2).

Please replace paragraph number [0157] with the following rewritten paragraph:

B24 [0157] Sections of the bursa of Fabricius of IBDV-infected chickens (Exp. 2) were examined after euthanization to determine the severity of bursa damage (Histopathological Bursa Lesions Score, HBLS). Bursae were classified according to Bayyari (Bayyari et al., 1996) from 1 for a normal bursa to 5 for 75-100% follicle damage (see Material and Methods). All viruses having the VP2 of CEF94 induced only minor bursa damage ( $< 2.0$ ), while all viruses having a VP2 of D6948 induced severe bursa damage ( $4.7 - 5.0$ ) (Table 13). Based upon the HBLS, it seems that bursa damage is almost identical after mD6948-s2VP3C1 and -s2VP3C3 infection, in comparison to unmodified D6948. However, careful examination reveals some clear differences between the induced damage of the bursa follicles by the different D6948-based viruses (~~Fig. 14~~). (FIG. 17). Both D6948 and rD6948 completely destroyed the follicular structure in the bursa and induced necrosis and cystic formation. The overall follicular structure of the bursas of mD6948-s2VP3C1- and -s2VP3C3-infected chickens is, however, still present. Some follicles of the mD6948-s2VP3C1-infected bursa have the same appearance as found in control bursas (PBS group), although the diameter of these follicles is generally reduced in comparison to wild-type follicles (see ~~Fig. 14~~). FIG. 17). We also found that the length of the bursa villi is reduced in (m)D6948-infected chickens, in comparison to mock-infected chickens. The reduction in the length of the villi in (m)D6948-infected bursas correlates with the reduced weight (and volume) of these bursas.

Please replace paragraph number [0162] with the following rewritten paragraph:

B25 [0162] In this example, we described the rescue of very virulent derived mosaic IBDV (mD6948-s2VP3C3), which has a VP3 that is based partly (the C-terminal 155 amino acids) upon the VP3 sequence of the serotype II TY89 strain. Earlier we described the generation and *in vitro* analysis of a mosaic virus (mCEF94-s2VP3C), based upon the classical attenuated serotype I strain CEF94, which has the same C-terminal exchange of the VP3 coding sequence (the C-terminal 155 amino acids) as mD6948-s2VP3C3. Replication of this virus appeared to be slightly reduced, as no virus release was found at 10 h post-infection of a monolayer of QM5

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cells. This is in contrast to wild-type CEF94, which showed a virus release at 10 h p.i. Equal final IBDV titers ( $> 10^7$ ) were reached both for wild-type CEF94 and mCEF94-s2VP3C between 25 and 50 h p.i., indicating that replication of mCEF94-s2VP3C is only minorly affected by the exchange of the C-terminal 155 amino acids of VP3 for those of TY89. Here we show that mD6948-s2VP3C3 has a reduced virulence in comparison to wild-type D6948. This conclusion is based upon the fact that i) it does not induce any mortality when administered to 21-day-old layer-type SPF chicks, while wild-type D6948 induced mortality in about 50% of the birds (Table 13), ii) the induced bursa damage after infection of 21-day-old SPF-chickens with mD6948-s2VP3C3 is reduced in comparison to infection with D6948 (~~Fig. 14~~). (FIG. 17). The introduction of the C-terminal part of VP3 serotype II in the background of a serotype I isolate leads apparently both *in vivo* and *in vitro* small but distinct phenotype.

Please replace paragraph number [0164] with the following rewritten paragraph:

B26

[0164] Sera of animals of experiment 2 were used in a competition Elisa, in which we used prokaryotically expressed C-terminal 290 amino acids of the CEF94 polyprotein as the coated antigen. Sera chickens infected with D6948 and mD6948-s2VP3C1 were used to compete with either monoclonal antibodies IV or I/G4. At a dilution of 1:500 of the chicken sera, we found that sera from chickens infected with mD6948-s2VP3C1 were not able to compete with of Mab IV and I/G4 in the interaction with the coated antigen, while sera of D6948-infected chickens were able to compete with this interacting (~~Fig. 15~~). (FIG. 18). This indicates that the introduction of the serotype II sequences into the cDNA of D6948 leads to a mosaic virus which induces a unique antibody response. Using the described competition Elisa, we are able to distinguish between chickens infected with the wild-type serotype I vvIBDV virus (strain D6948) and the mosaic serotype I-II, attenuated vvIBDV virus (strain mD6948-s2VP3C1). Despite the fact that the I/G4 did not react in the Western blot analysis with VP3 derived from pHB60 (~~Fig. 12~~), (FIG. 15), we found that antibodies induced by the derived virus of pHB60 (rD6948) were able to compete for the CEF94 antigen. The fact that the mosaic very virulent D6948 isolate (mD6948-s2VP3C3) has a reduced virulence (attenuated), combined

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with the ability of this virus to infect young chicks which have low, intermediate, or high levels of maternally-derived IBDV-neutralizing antibodies makes this virus a prime candidate for an efficacious vaccine to prevent outbreaks of vvIBDV. The ability to discriminate between chickens vaccinated (infected) with this virus from chickens infected with wild-type (very virulent) viruses makes this mosaic (vv)IBDV virus even more valuable, as it can be used to detect and follow (sub)-clinical (vv)IBDV infections in vaccinated flocks using our described competition Elisa. This (vv)IBDV marker vaccine also enables Gumboro eradication programs.

Please replace paragraph number [0166] with the following rewritten paragraph:

B27

[0166] Infectious Bursal Disease Virus (IBDV) is the causative agent of a highly contagious disease among chickens known as Gumboro disease (11). IBDV is a member of the family of *Birnaviridae*, having a double-stranded RNA (dsRNA) genome divided over two segments (14). The dsRNA genome is covered by a capsid of two viral proteins, which results in a single-shelled naked virus particle (60 nm) with an icosahedral (T=13) symmetry (5). The largest dsRNA segment (A-segment, about 3260 bp) contains two partly overlapping open reading frames (ORFs). The first, smallest ORF encodes the non-structural Viral Protein 5 (VP5, +/- 145 amino acids, 17 kDa, see Fig. 16A). ~~FIG. 19).~~ The second ORF encodes a polyprotein (1012 amino acids, 110 kDa, see Fig. 16B). ~~FIGs. 20A and 20B).~~ which is autocatalytically cleaved to yield the viral proteins pVP2 (also known as VPX, 48 kDa), VP4 (29 kDa) and VP3 (33 kDa) VP2, VP3, and VP4. During *in vivo* virus maturation, pVP2 is processed into VP2 (41-38 kDa), probably resulting from site-specific cleavage of the pVP2 by a host cell-encoded protease (19). VP2 and VP3 are the two proteins that constitute the shell of the virion. Neutralizing antibodies are only known for VP2, and these antibodies are conformation dependent. The B-segment (about 2827 bp) contains one large ORF, encoding the 91 kDa VP1 protein (see Fig. 16C). ~~FIGs. 21A and 21B).~~ This protein contains a consensus RNA-dependent RNA polymerase motif (8). Furthermore, this protein has been reported to be linked to the 5'-ends of the genomic RNA segments (Viral Protein genome-linked, VPg) (12, 29).



Please replace paragraph number [0170] with the following rewritten paragraph:

B28 [0170] To produce full-length single-stranded cDNA of both the A- and B-segments of the two IBDV isolates (CEF94 and D6948), we used primers specific for the 3'-end of the coding strand for reverse transcription (4). Two primers specific for the 3'-end of both the coding and non-coding strand were subsequently used to amplify the full-length A- and B-segment cDNAs in a PCR amplification using a mixture of *Taq* and *Pwo* enzymes (Expand, Boehringer Mannheim) (4). The two primers which hybridize with the 3'-terminus of the non-coding strand contained a 5' extension encoding the T7 promoter (4). Three independent RT-PCR reactions were performed for each segment and the resulting PCR fragments were cloned into the pGEM-T vector (Promega) (A-segment) or in a pUC19 derivative which contained the antigenomic cis-acting Hepatitis Delta Virus (HDV) ribozyme (10) and a T7 polymerase terminator (see Fig. 17- FIG. 22 and (3) (B-segment). Sequence analysis was performed on both strands using sequence-specific primers in a cycle sequencing reaction (BigDye terminator kit, PE Applied Biosystems) and an ABI310 apparatus (PE Applied Biosystems). An unintended mutation in one of the A-segment of D6948 clones (pHB-22) was restored by exchanging a restriction enzyme fragment from an independent clone (data not shown), yielding pHB-22R. pHB-22R contains the consensus cDNA of the A-segment of the D6948 vvIBDV isolate. We subsequently transferred this full-length A-segment sequence into a pUC18-based vector, which contained the Hepatitis Delta Virus (HDV) ribozyme and a T7 polymerase terminator (see above) by PCR amplification using the same primers as used in the RT-PCR protocol (4). During this transfer, an unintended mutation was introduced in the VP4-encoding part of the polyprotein (A1817G). This mutation was subsequently used as a genetic tag for virus rescued from this D6948 A-segment plasmid. The A-segment cDNA clone of CEF94 (pHB-36W) contains a two-nucleotide genetic tag (3172<sub>C→T</sub> and 3173<sub>T→A</sub>), thereby introducing a unique *KpnI* restriction site GGTAAC (SEQ ID NO:1) in the 3'-UTR of the A-segment (3).

Please replace paragraph number [0176] with the following rewritten paragraph:

B29 [0176] To replace the coding sequence of the pVP2 part of the CEF94 polyprotein with the corresponding part of the D6948 polyprotein, we generated three PCR fragments (i.e., VP2a, VP2b and VP2c, see ~~Fig. 21~~). FIG. 26. PCR fragment VP2a (189 bp) was generated using primers M13R (TCACACAGGAAACAG CTATGAC (SEQ ID NO:16)) and ATG3 (CATCGCTGCGATCGTTTGTCTGATCTCTAC (SEQ ID NO:17)), and pHB-36W as template. PCR fragment VP2b (761 bp) was generated by using primers (ATCCGGGGCCCTAAGGAGG (SEQ ID NO:18)) and ANC4 (GCCAAGTCGGTGTGCAG (SEQ ID NO:19)), and pHB-36W as template. PCR fragment VP2c (1418 bp) was generated using primers HY0P (TATCATTGATGGTCAGTAGAG (SEQ ID NO:20)) and HY2M (CACCGGCACAGCTATCC (SEQ ID NO:21)), and pHB-22R as template. These three PCR fragments were agarose gel purified using a Qiaex gel extraction kit (Qiagen, Germany) and used as template (50 ng of each fragment) in a fusion PCR using primers T7EcoRI (GGAATTCTAATACGACTCACTATAGG (SEQ ID NO:22)) and ANC4 (SEQ ID NO:19). This PCR fragment (2256 bp) was subsequently digested with *EcoRI* and *SacII* (resulting in a 1806 bp fragment), agarose gel purified (Qiaex gel extraction kit, Qiagen) and ligated into pHB-36W, which had been digested with the same restriction enzymes, and was then used to transform *E.coli* DH5a cells. Several plasmids were analyzed and one plasmid having the intended sequence was selected (pHB36-vvVP2).

Please replace paragraph numbers [0182] and [0183] with the following rewritten paragraphs:

B30 [0182] To study molecular determinants that are responsible for the very virulent phenotype of vvIBDV isolates, we cloned and sequenced the full-length A- and B-segments of the very virulent IBDV isolate D6948 three times independently. The cDNA of the D6948 A-segment (3260 bp) differs at 122 positions from the sequence of the classical, attenuated CEF94 IBDV isolate (data not shown). These nucleotide differences result in an N-terminal extension of 4 amino acids (MLSL) for the predicted VP5 of D6948, and 5 additional amino acid

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differences between the two VP5 proteins (~~Fig. 16A~~) (FIG. 19). Furthermore, 18 amino acid differences are present between the two polyproteins, of which 11 are located in the pVP2 part, 5 in the VP4 part, and 2 in the VP3 part of the polyprotein (~~Fig. 16B~~) (FIGs. 20A and 20B). Between the two B-segments (2827 bp), we found 288 nucleotide differences (data not shown), resulting in 17 amino acid differences between the two VP1's (~~Fig. 16C~~) (FIGs. 21A and 21B), the only protein encoded on the B-segment.

[0183] By combining sequences of different full-length cDNA clones, we constructed plasmids which contained the consensus cDNA sequence of the D6948 A- and B-segments. The A- and B-segment cDNAs, including an artificially introduced T7 promoter sequence, were subsequently transferred to a pUC19-based vector which contained a cis-acting hepatitis delta virus ribozyme (10), yielding pHB-60 (A-segment) and pHB-55 (B-segment) (Table 16 and ~~Fig. 17~~ FIG. 22). These pUC19-based transcription plasmids are the basis for rescuing rIBDV from cloned cDNA, using a recombinant helper virus (Fowlpox) that expresses T7 polymerase (6). We have used this *in vivo* T7 expression system previously to rescue infectious IBDV from cloned cDNA of the cell culture adapted, classical IBDV isolate CEF94 (3).

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Please replace paragraph number [0184] with the following rewritten paragraph:

B31

[0184] The A- and B-segment cDNA clones of vvIBDV isolate D6948 were used as templates in an *in vitro* transcription/translation reaction (TnT-T7Quick, Promega). The protein products of these cDNA clones appeared to be identical to those of the classical attenuated CEF94 cDNA plasmids (i.e., pVP2, VP3, and VP4 in case of the A-segment and VP1 in case of the B-segment) in an SDS-PAGE analysis (~~Figs. 18a and 18b~~ FIGs. 23A and 23B). The pVP2 from D6948 is found at a slightly higher position than the pVP2 protein from CEF94. Plasmid pHB-60 (D6948 A-segment) contains a single nucleotide substitution at position 1817 nt (A1817G), resulting from the PCR-based cloning strategy. This mutation leads to a conserved amino acid substitution in VP4 (I563V). As this mutation did not affect the processing of the polyprotein (~~Fig. 18a~~) (FIG. 23A and unpublished results), we have subsequently used this mutation as a tag for virus rescued from this D6948 A-segment plasmid.

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Please replace paragraph number [0185] with the following rewritten paragraph:

B32  
[0185] Plasmids containing the A- and B-segment cDNA of either CEF94 or D6948 were used to co-transfect QM5 cells. Transient expression of the viral proteins originating from both the A- or B-segments was observed in all examined cases in an immunoperoxidase monolayer assay (IPMA), by using antibodies specific for either VP3/4 (A-segment) or VP1 (B-segment) (data not shown). To assess the production of infectious IBDV originating from cloned cDNA (referred hereafter as rescued IBDV; rIBDV), we transferred part of the supernatant onto a fresh monolayer of QM5 cells (first passage) and analyzed the expression of viral proteins in these cells after 24h in an IPMA. This analysis showed that no infectious rIBDV could be detected in QM5 cells treated with the supernatant of a co-transfection of the A- and B-segment plasmids of vvIBDV isolate D6948 (see ~~Fig. 19~~ FIG. 24). In contrast, infectious rIBDV was present in QM5 cells treated with supernatant of the co-transfection with plasmids containing the A- and B-segments of the attenuated CEF94 isolate (~~Fig. 19~~ FIG. 24). The co-transfection of the A-segment plasmid of D6948 with the B-segment plasmid of CEF94 yielded no infectious segment-reassorted IBDV (srIBDV-DACB, Table 16). However, the reciprocal combination srIBDV-CADB (Table 16) did yield infectious segment-reassorted IBDV (~~Fig. 19~~ FIG. 24). Wild-type IBDV isolates such as D6948 are unable to grow on fibroblast cells like QM5 (see Introduction). Therefore, we assessed the presence of infectious rD6948 originating from cloned cDNA by transferring supernatants of transfected QM5 cells onto primary bursa cells which had been grown *in vitro* for 24h. Similar to wild-type D6948, which can infect only the lymphoid cells present in this monolayer (data not shown), we were able to detect rD6948 in several lymphoid cells after two days of incubation with the transfection supernatant (~~Fig. 19~~ FIG. 24, lower panel). Incubation of primary bursa cells with the transfection supernatant of A- and B-segment cDNAs from CEF94 showed that not only lymphoid cells but also fibroblast cells were infected, leading to a destruction of the monolayer within 48h of incubation. Virus present in the supernatant of a transfection with the A-segment cDNA of D6948 and B-segment cDNA of CEF94 was only able to infect lymphoid cells, and no destruction of the monolayer was observed after 48h. Similar to rD6948, srIBDV-DACB is

B32 cont'd  
 apparently unable to infect fibroblast cells. Again, infection of fibroblast cells was found with virus originating from transfection of the A-segment cDNA of CEF94 and B-segment cDNA of D6948, although the monolayer appeared to be less destructed after 48h in comparison with rCEF94 (~~Fig. 19~~). (FIG. 24).

Please replace paragraph number [0186] with the following rewritten paragraph:

B33  
 [0186] To determine whether the rescued rCEF94 and srIBDV-CADB had the same replication properties on QM5 cells as wild-type CEF94, we performed single-step growth curves (in triplicate) for each of these viruses. QM5 cells were infected during one hour with IBDV (m.o.i. = 5), after which time the cells were rinsed three times and incubated in complete medium. Part of the supernatant of the infected QM5 cultures was removed at different time points. The amount of IBDV (TCID<sub>50</sub> per ml) was determined in each sample (~~Fig. 20~~). (FIG. 25). Release of infectious IBDV was found at 10h p.i. for both wild-type CEF94 and rCEF94, while the first release of srIBDV-CADB was only found at 15h p.i. Final titers (24h p.i.) were about the same for wild-type CEF94, rCEF94, and the rescued segment-reassorted IBDV containing the A-segment of CEF94.

Please replace paragraph number [0187] with the following rewritten paragraph:

B34  
 [0187] To analyze the viral determinants responsible for the very virulent phenotype of isolate D6948, we constructed three different plasmids containing full-length mosaic IBDV A-segment cDNA. Using a PCR-based method (~~see Fig. 21~~), FIG. 26), we constructed DNA fragments of which the middle part consisted of cDNA originating from D6948 (i.e., the pVP2-, VP3- or VP4-encoding part), while the flanking cDNA originated from CEF94. The transition positions between the different cDNAs are at the putative cleavage sites between the pVP2-VP4 and VP4-VP3 parts of the polyprotein (i.e., behind amino acids 453 and 723, respectively (18), ~~see Fig. 16B~~). FIGs. 20A and 20B). The mosaic PCR fragments were subsequently used to replace the corresponding part of CEF94 A-segment cDNA in plasmid pHB-36W, using specific restriction endonucleases. The exchanged parts of the generated plasmids, named

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pHB36-vvVP2, -vvVP3, and -vvVP4, were sequenced and plasmids containing the intended mosaic cDNA sequence were subsequently used for *in vitro* transcription/translation and analyzed for the production of viral proteins by means of SDS-PAGE (~~Fig. 18e~~) (FIG. 23C). No apparent difference in the autocatalytic cleavage was observed in the polyproteins encoded by these mosaic cDNA A-segments.

Please replace paragraph number [0188] with the following rewritten paragraph:

B35

[0188] The plasmids pHB36-vvVP2, -vvVP3, and -vvVP4 were used to transfect QM5 cells together with the B-segment cDNA of CEF94 (pHB-34Z). The presence of infectious IBDV (hereafter referred to as mIBDV, see also Table 16) was analyzed by transferring aliquots of the transfection supernatant to a fresh monolayer of QM5 cells. After 1 day of incubation, we fixed the cells and used an IBDV-specific antibody assay (IPMA) to test for the presence of mIBDV. No mIBDV could be detected in the QM5 monolayer when supernatant of the pHB36-vvVP2 plasmid was used, while in the case of pHB36-vvVP3 and pHB36-vvVP4, mIBDV was clearly present (~~Fig. 22~~) (FIG. 27). The fact that we did not detect mCEF94-vvVP2 in the QM5 monolayer could result from the difference in cell tropism between the rCEF94 and mCEF94-vvVP2. To check for this possibility, we transferred part of the supernatant of the transfection with pHB36-vvVP2 onto a monolayer of primary bursa cells. After two days, we were able to show the presence of mCEF94-vvVP2 virus in some of the B-lymphoid cells (~~Fig. 22~~, FIG. 27, lower panel). Apparently, mCEF94-vvVP2 had the same cell-tropism as D6948, rD6948, and srIBDV-DACB, since it was only able to grow on B-lymphoid cells and not on fibroblast cells.

Please replace paragraph numbers [0194] through [0197] with the following rewritten paragraphs:

B36

[0194] After having verified that the deduced cDNA sequence of D6948 represents indeed a true vvIBDV sequence, we compared the deduced amino acid sequence of D6948 proteins with those of other (vv)IBVD isolates. One striking difference between the VP5

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sequences of the classical attenuated isolate CEF94 and the vvIBDV isolate D6948 is that the vvIBDV VP5 has an N-terminal extension of 4 amino acids (see ~~Fig. 16A~~, FIG. 19). This extension is also predicted for the two other vvIBDV isolates of which the sequences of this region have been published (HK46 (20) and UK661 (7)). All classical isolates lack this N-terminal extension, while the only antigenic variant (GLS) of which the cDNA sequence in this region is available has the same extension. Whether the AUG-codon at position 85 is indeed used as a start codon for the production of VP5 of the very virulent and the GLS isolate remains to be determined. It would be interesting to see if the N-terminal extended VP5 results in a different phenotype. Of the other amino acid differences in VP5 (~~Fig. 16A~~), (FIG. 19), only R49G and W137R are found for all known vvIBDV VP5 sequences (UK661, OKYM (33), and HK46). Several reports have focused on differences between the pVP2 sequences of IBDV isolates of different origin (e.g., (15)). The amino acid difference at positions 253, 279, 284, and 330 (~~Fig. 16B~~) (FIGs. 20A and 20B) are most likely the result of adaptation of CEF94 on CEF cells (20, 22, 33). Of the remaining amino acid differences, only 8 are conserved (see amino acid residues in bold face, ~~Fig. 16B~~) FIGs. 20A and 20B) among all the four vvIBDV isolates (D6948, UK661, OKYM, and HK46), compared with the published sequences of classical and antigenic variant isolates.

[0195] The length of the ORF for VP1/VPg of CEF94 is two amino acids longer than the same ORF of D6948. Heterogeneity in the length of the VP1/VPg ORF has also been found by others (34). All VP1/VPg's of vvIBDV isolates appear to have a size of 879 amino acids, while some of the VP1's of classical isolates have a size of only 877 amino acids. The 881 amino acids found for the VP1/VPg of CEF94 is the largest VP1 published so far. Furthermore, we identified 17 amino acid differences between the VP1/VPg sequences of CEF94 and D6948 (~~Fig. 16C~~) (FIGs. 21A and 21B). Although it has been found that attenuation of IBDV results in amino acid changes in the VP1 sequence (34), no direct correlation between the three changes identified by Yehuda et al. and attenuation has been proven. In fact, we show that segment-reassorted IBDV (srIBDV-DACB, see Table 16), which contains the B-segment of a classical, attenuated IBDV isolate, induces the same mortality and histopathologic lesions as

wild-type very virulent IBDV. This indicates that the amino acid changes found in VP1 after adaptation are not related to the attenuation of the virus. Six of the 17 amino acid differences are found in all of the vvIBDV isolates of which the cDNA sequence has been published (see amino acid residues in bold face in ~~Fig. 16C~~ FIGs. 21A and 21B).

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[0196] To assess the influence of the amino acid differences between the viral proteins from different isolates, we constructed plasmids which contained cDNA from both CEF94 and D6948. A small difference in molecular weight between the pVP2 proteins encoded by the cDNA of CEF94 and D6948 was found in an SDS-PAGE analysis (~~Fig. 18a~~) (FIG. 23A). The difference in position in the SDS-PAGE gel is also found for the pVP2 proteins encoded by pHB36W and pHB36-vvVP2 (~~Fig. 18e~~) (FIG. 23C). A small difference is present between the position of VP4 encoded by pHB36-vvVP3 and pHB-36-vvVP4, in comparison with VP4 encoded by pHB-36W. The first amino acid difference between the two VP3 proteins of CEF94 and D6948 (~~H751D, Fig. 16B~~) FIGs. 20A and 20B) is found 28 amino acids downstream of the proposed cleavage site (behind amino acid 722 (18), see ~~Fig. 16B~~) FIGs. 20A and 20B). The difference in the molecular weight of VP4 can be explained if the actual cleavage site of VP4-VP3 is not behind the two basic amino acid residues, but at least >28 amino acids more downstream. Alternative cleavage sites between VP4/VP3 have been proposed to be located behind amino acid 736 (after Tyr-Leu (13) or in the region 752-756 (A-X-A-A-S, (18)). Recently it was indeed shown, using a site-directed mutagenesis approach, that the cleavage site between VP4 and VP3 is most likely located between amino acid residues 755 and 756 (27). We observed that polyclonal antibodies raised against part of the polyprotein starting at position 722 is also able to react strongly with VP4, adding further proof to the assumption that the actual cleavage site is located downstream of amino acid 722 (Boot unpublished data).

[0197] The viruses derived from the mosaic A-segment plasmids were checked for growth on either QM5 cells and/or primary bursa cells and used to challenge SPF chickens. Segment-reassorted virus containing the A-segment of D6948 and the B-segment of CEF94 (srIBDV-DACB, Table 16) induced the same signs and lesions as wild-type and recombinant D6948 (Table 17). The differences found within the VP1/VPg proteins of classical (adapted) and



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very virulent (non-adapted) isolates have apparently no major influence on virulence. The reciprocal combination (srIBDV-DACB, Table 16) induced less CPE in the monolayer of primary bursa cells in comparison to rCEF94 (~~Fig. 19~~) (FIG. 24). We also found a delay in release of new viral particles by srIBDV-CADB in comparison with CEF94 and rCEF94 (~~Fig. 20~~) (FIG. 25). Despite the difference in *in vitro* virus release between these isolates, we found no difference in the *in vivo* challenge using SPF chickens. IBDV antibody titers of rCEF94 and srIBDV-CADB are comparable both at 9 days p.i. (Table 17) and 15 days p.i. (data not shown). It has been suggested that segment reassortment between B-segments of serotype I and II isolates occurs in the field (7). Naturally occurring segment reassorting is frequently found for highly segmented dsRNA viruses (Reo- and Rotavirus, (26)). We did not find any phylogenetic evidence for a natural reassortment of IBDV segments. All VP1 proteins of vvIBDV have distinct amino acid changes when compared to classical serotype I isolates and serotype II isolates (~~Fig. 16C~~, FIGs. 21A and 21B, and data not shown). Although no proof exists for IBDV reassortments in the field, Müller has succeeded in producing segment-reassorted virus in the laboratory by co-cultivation of strains belonging to the different serotypes (21).

Please replace Table 4 (designated paragraph number [0203]) with the following rewritten Table 4:

[0203] Table 4 Description of the used plasmids

| Name         | Based on plasmid | Description  |
|--------------|------------------|--|
| pUC18-Ribo   | pUC18            | Contains the <i>SmaI-XbaI</i> fragment of pTV-2A   |
| pHB-36A      | pUC18-Ribo       | Contains the consensus cDNA sequence of the A-segment of CEF94 (see Fig. 2a <u>FIGs. 2A-2G</u> )   |
| pHB-36W      | pHB-36A          | An artificially introduced <i>KpnI</i> -site (genetic tag) in the 3'-UTR of the CEF94 A-segment-encoding cDNA ( <u>Fig. 2</u> ) (see <u>FIGs. 2A-2G</u> and <u>3A-3F</u> ) |
| pHB-36       | pHB-36A          | Contains a lethal amino acid substitution in the VP4 part of the polyprotein (V582A)   |
| pHB-60       | pUC18-Ribo       | Contains the consensus cDNA sequence of the D6948 A-segment (see Fig. 2a) <u>FIGs. 2A-2G</u> )   |
| pHB-34Z      | pUC18-Ribo       | Contains the consensus cDNA sequence of the CEF94 B-segment (see Fig. 2b) <u>FIGs. 3A-3F</u> )   |
| pHB-55       | pUC18-Ribo       | Contains the consensus cDNA sequence of the D6948 B-segment (see Fig. 2b) <u>FIGs. 3A-3F</u> )   |
| pSV-TY89-VP3 | pGEM-Teasy       | Contains the consensus cDNA of TY89 encoding the entire VP3 (A-segment, see Fig. 2) <u>FIGs. 2A-2G</u> and <u>3A-3F</u> )  |
| pHB36-vvVP2  | pHB-36W          | Contains D6948 A-segment cDNA which encodes the entire VP2 (453 amino acids)   |
| pHB36-vvVP3  | pHB-36W          | Contains D6948 A-segment cDNA which encodes the entire VP3 (289 amino acids)   |

|               |         |   |
|---------------|---------|---|
| pHB36-vvVP4   | pHB-36W | Contains D6948 A-segment cDNA which encodes the entire VP4 (270 amino acids)  |
| pHB36-s2VP3   | pHB-36W | Contains TY89 A-segment cDNA which encodes the entire VP3 (289 amino acids)   |
| pHB36-s2VP3C  | pHB-36W | Contains TY89 A-segment cDNA which encodes the C-terminal part (122 amino acids) of VP3   |
| pHB36-s2VP3N  | pHB-36W | Contains TY89 A-segment cDNA which encodes the N-terminal part (168 amino acids) of VP3   |
| pHB60-s2VP3C1 | pHB-60  | Contains cDNA encoding a mosaic polyprotein (D6948 (1-543 AA), CEF94 (544-889 AA), and TY89 (890-1012 AA)). The 5'-UTR is derived from D6948, while the 3'-UTR is derived from CEF94. A unique <i>Kpn</i> I-site (genetic tag) is furthermore present in the 3'-UTR |